

ATTACHMENT A

FRESHWATER ACUTE  
TOXICITY TEST PROCEDURE AND PROTOCOL

I. GENERAL REQUIREMENTS

The Permittee shall conduct acceptable acute toxicity tests in accordance with the appropriate test protocols described below:

- Daphnid (Ceriodaphnia dubia) definitive 48 hour test.
- Fathead Minnow (Pimephales promelas) definitive 48 hour test.

Acute toxicity data shall be reported as outlined in Section VIII.

II. METHODS

Methods to follow are those recommended by EPA in:

Weber, C.I. et al. *Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms*, Fourth Edition. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, August 1993, EPA/600/4-90/027F.

Any exceptions are stated herein.

III. SAMPLE COLLECTION

A discharge sample shall be collected. Aliquots shall be split from the sample, containerized and preserved (as per 40 CFR Part 136) for chemical and physical analyses required. The remaining sample shall be measured for total residual chlorine and dechlorinated (if detected) in the laboratory using sodium thiosulfate for subsequent toxicity testing.

**(Note that EPA approved test methods require that samples collected for metals analyses be preserved immediately after collection.)** Grab samples must be used for pH, temperature, and total residual chlorine (as per 40 CFR Part 122.21).

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*Standard Methods for the Examination of Water and Wastewater* describes dechlorination of samples (APHA, 1992).

Dechlorination can be achieved using a ratio of 6.7 mg/L anhydrous sodium thiosulfate to reduce 1.0 mg/L chlorine. A thiosulfate control (maximum amount of thiosulfate in lab control or receiving water) should also be run.

All samples held overnight shall be refrigerated at 4°C.

**IV. DILUTION WATER**

Grab samples of dilution water used for acute toxicity testing shall be collected from the receiving water at a point upstream of the discharge free from toxicity or other sources of contamination. Avoid collecting near areas of obvious road or agricultural runoff, storm sewers or other point source discharges. An additional control (0% effluent) of a standard laboratory water of known quality shall also be tested.

If the receiving water diluent is found to be, or suspected to be toxic or unreliable, an alternate standard dilution water of known quality with a hardness, pH, conductivity, alkalinity, organic carbon, and total suspended solids similar to that of the receiving water may be substituted **AFTER RECEIVING WRITTEN APPROVAL FROM THE PERMIT ISSUING AGENCY(S)**. Written requests for use of an alternate dilution water should be mailed with supporting documentation to the following address:

Director  
Office of Ecosystem Protection  
U.S. Environmental Protection Agency New England  
1 Congress Street  
Mail Code: CPE  
Boston, Massachusetts 02214-2023

It may prove beneficial to the Permittee to have the proposed dilution water source screened for suitability prior to toxicity testing. EPA strongly urges that screening be done prior to set up of a full definitive toxicity test any time there is question about the dilution water's ability to support acceptable performance as outlined in the 'test acceptability' section of the protocol. See Section 7 of EPA/600/4-89/001 for further information.

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## V. TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

The following two tables summarize the accepted Daphnid and Fathead Minnow toxicity test conditions and test acceptability criteria:

**EPA NEW ENGLAND RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS  
FOR THE DAPHNID, *Ceriodaphnia dubia*, 48 HOUR ACUTE TEST <sup>1</sup>**

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|-----|--|--|
| 1.  | Test type:                                       | Static, non-renewal  |
| 2.  | Temperature (°C):                                | 25 ± 1°C   |
| 3.  | Light quality:                                   | Ambient laboratory illumination  |
| 4.  | Photo-period                                     | 16 hr. light, 8 hr. dark   |
| 5.  | Test chamber size:                               | 30 ml  |
| 6.  | Test solution volume:                            | 25 ml  |
| 7.  | Age of test organisms:                           | 1-24 hours (neonates)  |
| 8.  | Number of daphnids per test chamber:             | 5  |
| 9.  | Number of replicate test chambers per treatment: | 4  |
| 10. | Total number of daphnids per test concentration: | 20   |
| 11. | Feeding regime:                                  | Feed YCT and <i>Selenastrum</i> while holding organisms prior to initiating tests as per manual. |
| 12. | Aeration:  | None   |

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13. Dilution water<sup>2</sup>: Receiving water, other surface water, synthetic soft water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q® or equivalent deionized water and reagent grade chemicals according to EPA chronic toxicity test manual) or deionized water combined with mineral water to appropriate
14. Dilution factor: > 0.5
15. Number of dilutions<sup>3</sup>: 5 plus a control. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution series.
16. Effect measured: Mortality; no movement of body or appendages on gentle prodding.
17. Test acceptability: 90% or greater survival of test organisms in the control solutions.
18. Sampling requirements: For on-site tests, samples are collected daily and used within 24 hr. of the time they are removed from the sampling device. For off-site tests, samples must be first used within 36 hours of collection.
19. Sample volume required: Minimum 1 liter.

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**Footnotes:**

- (1) Adapted from EPA/600/4-90/027F.
- (2) Standard prepared dilution water must have hardness requirements to generally reflect characteristics of the receiving water.
- (3) When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

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**EPA NEW ENGLAND RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR  
THE FATHEAD MINNOW, *Pimephales promelas*, 48 HOUR ACUTE TEST<sup>1</sup>**

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|---|--|
| 1. Test type:                                   | Static, non-renewal  |
| 2. Temperature (°C):                            | 20 ± 1°C or 25 ± 1°C   |
| 3. Light quality:                               | Ambient laboratory illumination  |
| 4. Photo-period:                                | 16 hr. light, 8 hr. dark   |
| 5. Test chamber size:                           | 250 ml minimum   |
| 6. Test solution volume:                        | Minimum 200 ml/replicate   |
| 7. Age of test organisms:                       | 1-14 days old and age within 24 hrs of all other fish  |
| 8. No. organisms per chamber:                   | 10 (not to exceed loading limits)  |
| 9. No. of replicate test vessels per treatment: | 4  |
| 10. Total no. organisms per concentration:      | 40   |
| 11. Feeding regime:                             | Light feeding using concentrated brine shrimp nauplii while holding prior to initiating the test as per manual.  |
| 12. Aeration:                                   | None, unless dissolved oxygen (D.O.) concentration falls below 4.0 mg/L, at which time gentle single bubble aeration should be started at a rate of less than 100 bubbles/min (Routine D.O. check is recommended). |

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|------------------------------------|--|
| 13. Dilution water: <sup>2</sup>   | Receiving water, other surface water, synthetic soft water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q® or equivalent deionized and reagent grade chemicals according to EPA chronic toxicity test manual) or deionized water combined with mineral water to appropriate hardness. |
| 14. Dilution factor:               | ≥ 0.5  |
| 15. No. of dilutions: <sup>3</sup> | 5 plus a control. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution series.  |
| 16. Effect measured                | Mortality; no movement of body or appendages on gentle prodding.   |
| 17. Test acceptability:            | 90% or greater survival of test organisms in control solution.   |
| 18. Sampling requirements:         | For on-site tests, samples are collected daily and used within 24 hr. of the time they are removed from the sampling device. For off-site tests, samples must be first used within 36 hours of collection.   |
| 19. Sample volume required:        | Minimum 2 liters   |

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**Footnotes:**

- (1) Adapted from EPA/600/4-90/027F.
- (2) Standard dilution water must have hardness requirements to generally reflect characteristics of the receiving water.
- (3) When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

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## VI. CHEMICAL ANALYSIS

At the beginning of a static acute toxicity test, pH, conductivity, total residual chlorine, and temperature must be measured in the highest effluent concentration and the dilution water. Dissolved oxygen, pH and temperature are also measured at 24- and 48-hour intervals. It is also recommended that total alkalinity and total hardness be measured in the control and highest effluent concentration at the beginning of the test. The following chemical analyses shall be performed for each sampling

<u>Parameter</u>	<u>Effluent</u>	<u>Diluent</u>	<u>Min. Quantification Level (mg/l)</u>
Hardness <sup>1</sup>	X	X	0.5
Alkalinity	X	X	2.0
pH	X	X	--
Specific Conductance	X	X	--
Total Solids & Suspended Solids	X	X	--
Ammonia	X	X	0.1
Total Organic Carbon	X	X	0.5
Total Residual Chlorine (TRC) <sup>2</sup>	X	X	0.05
Dissolved Oxygen	X	X	1.0
<u>Total Metals</u>			
Cd	X		0.001
Cr	X		0.005
Pb	X	X	0.005
Cu	X	X	0.0025
Zn	X	X	0.0025
Ni	X	X	0.004
Al	X	X	0.02
Mg, Ca	X	X	0.05

## Footnotes:

- (1) Method 2340 B (hardness by calculation) from APHA (1992), *Standard Methods for the Examination of Water and Wastewater*, 19th or subsequent Edition(s) as approved in 40 CFR Part 136.

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## (2) Total Residual Chlorine

Either of the following methods from APHA (1992), *Standard Methods for the Examination of Water and Wastewater*, 19th or subsequent Edition(s) as approved in 40 CFR Part 136 must be used for these analyses:

- Method 4500-Cl E. Low-Level Amperometric Titration (the preferred method); or
- Method 4500-Cl G. DPD Colorimetric Method, or use U.S. EPA *Manual of Methods Analysis of Water and Wastes* Method 330.5.

## VII. TOXICITY TEST DATA ANALYSIS

LC50 Median Lethal Concentration (Determined at 48 Hours)

Methods of Estimation:

- Probit Method
- Spearman-Kärber
- Trimmed Spearman-Kärber
- Graphical

See the flow chart in Figure 6 on p. 77 of EPA 600/4-90/027F for appropriate method to use on a given data set.

No Observed Acute Effect Level (NOAEL)

See the flow chart in Figure 13 on p. 94 of EPA 600/4-90/027F.

## VIII. TOXICITY TEST REPORTING

A report of results will include the following:

- Description of sample collection procedures, site description;
- Names of individuals collecting and transporting samples, times and dates of sample collection and analysis on chain-of-custody; and
- General description of tests: age of test organisms, origin, dates and results of standard toxicant tests; light and temperature regime; other information on test conditions if different than procedures recommended. Reference toxicant test data should be included.



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- All chemical/physical data generated. (Include minimum detection levels and minimum quantification levels.)
- Raw data and bench sheets.
- Provide a description of dechlorination procedures (as applicable).
- Any other observations or test conditions affecting test outcome.